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Highly conserved B-subunit genes of Shiga-like toxin II variants found in *Escherichia coli* O157 strains

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Abstract: To determine the degree of heterogeneity among Shiga-like toxin-II (SLT-II)-related toxins present in enterohemorrhagic *Escherichia coli* O157 strains, *slt-IIB*-related genes of 15 strains were amplified and sequenced. Of these 15 isolates, six contained only the *slt-II*-related genes, seven strains harbored *slt-II*-related genes together with *slt-II*, and two strains had *slt-II*-related genes plus *slt-I*. In strains carrying *slt-II*-related genes alone or in combination with *slt-I*, the PCR fragments were directly subjected to Taq cycle sequence analysis. Direct sequencing was not possible with the seven strains possessing both *slt-II* and *slt-II*-related genes, since the PCR products contained both genes. In order to allow sequence analysis of these *slt-II*-related genes, the PCR products were first subjected to restriction enzyme digestion with *Fok*I, which selectively digested *slt-IIB*. This resulted in an undigested 270-bp fragment consisting of pure *slt-II*-related genes. Interestingly, comparison of the nucleotide sequences revealed 100% homology of all analyzed 15 *slt-IIB*-related toxin genes. In addition, the nucleotide sequence of *slt-IIB*-related toxin genes were identical to *slt-IIcB*. Our findings indicate that SLT-IIc is a major variant form of SLT-II present in *E. coli* O157 strains.

Key words: Escherichia coli O157; Shiga-like toxin II variants; Nucleotide sequence analysis

Introduction

Shiga-like toxin (SLT, verocytotoxin)-producing *Escherichia coli* have been recognized to be associated with intestinal and extraintestinal diseases in humans and animals [1]. SLTs are bipartite molecules, comprising one A- and multiple B-subunits. The enzymatically active A-subunit function as RNA glycosidase and the B-subunits are responsible for binding the toxins to eukaryotic glycolipid receptors. SLTs can be divided into two major toxin types: Shiga-like toxin-I (SLT-I, VT1) and Shiga-like toxin-II (SLT-II, VT2). Molecular studies on SLT-I from different *E. coli* strains revealed that SLT-I are either fully identical to Shiga toxin of *Shigella dysenteriae* type I or differ by only one amino acid [2,3]. Members of the SLT-II group are not neutralized by anti-Shiga toxin and reveal no cross-hybridization with SLT-I-specific DNA probes. Toxins of the SLT-II family show sequence and antigenic variation. This has resulted in subgrouping of SLT-II toxins. One subgroup includes the prototype SLT-II encoded by bacteriophage 933W [4]

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and several variants of SLT-II (i.e. SLT-IIvha (VT2vha), SLT-IIvhb (VT2vhb), SLT-IIvhc, SLT-IIc) which are about 97% related to SLT-II at the deduced amino acid level of the B-subunits while the A-subunits share 98–100% homology with SLT-IIA [5–7]. These SLT-II-related toxins exhibit only partial serological reactivity to SLT-II and are now collectively designated as SLT-IIc (VT2c) [8]. A second subtype of the SLT-II family consists of SLT-IIe (SLT-IIv, VTe) which shares at the amino acid level of the A- and B-subunits 93.0% and 84.0% homology to SLT-II, respectively [9]. SLT-IIe is found in porcine *E. coli* strains and is associated with edema disease of pigs. A third SLT-II-related toxin is resembled by SLT-IIva which at the amino acid level shares only 71.4% homology in the A- and 84.3% in the B-subunit to SLT-II [10]. Recently additional toxins closely related to SLT-II have been described in *E. coli* isolated from patients with intestinal diseases, suggesting great heterogeneity among the SLT-II-related toxins [11–13]. Since major differences in biological activity correlate with differences in the B-subunit genes the purpose of this study was to determine the degree of heterogeneity among SLT-II-related toxins found in *E. coli* O157 a serogroup most often isolated from patients with hemolytic-uremic syndrome and hemorrhagic colitis [1,14].

Materials and Methods

Bacterial strains

The *E. coli* O157 strains used in this study, their origin and the diseases they caused are listed in Table 1. The strains were identified either by colony blot hybridization using oligonucleotide probes 772 and 849 as described [15] or by PCR with primers MK1/MK2 [16]. Probe 772 identifies *slt-I* and probe 849 *slt-II* and its variant forms [15]. Biochemical characterization revealed that all *E. coli* O157 strains gave a negative sorbitol and β-glucuronidase reaction. The control strains used in the *slt* genotyping studies and for the sequence analyses were the SLT-II-producing *E. coli* strain C600(933W), the *E. coli* strain C600(H19J) producing SLT-I and the SLT-IIc-producing *E. coli* strain E32511. The latter isolate has been shown to have lost its *slt-II* during subcultivation [6].

PCR for amplification of the B-subunit genes

The sequences of primers used to amplify the *slt-IIB* and *slt-IIB*-related genes were GK5 (5′-ATG AAG AAG ATG TTT ATG GCG-3′) and GK6 (5′-TCA GTC ATT ATT AAA CTG CAC-3′) [16], whereas primers KS7 and KS8 [17] were used to amplify *slt-IB*. Bacterial DNA was prepared by incubating 10 μl of bacterial suspension (10^4 bacteria) for 10 min at 95°C. Amplifications were performed in a total volume of 50 μl containing 200 μM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 30 pmol of each primer, 5 μl 10-fold concentrated polymerase synthesis buffer, and 2.5 U of Taq DNA Polymerase (Amersham Laboratories, Buckinghamshire, UK). The samples were incubated at 94°C for 30 s to denature the DNA, 1 min at 52°C to anneal the primers, and then at 72°C for 1 min to extend the annealed primers. After the last cycle the amplification products were subjected to submarine gel electrophoresis on 2% agarose gels, and visualized by staining with ethidium bromide.

Restriction enzyme analysis of the PCR products

To distinguish between *slt-IIB* and *slt-IIB*-related sequences following PCR with primers GK5 and GK6, restriction analysis of the PCR amplification products was carried out with *Hae*III and *Fok*I as described [19]. Briefly, 10-μl aliquots of the amplified products recovered after PCR were subjected to restriction endonuclease digestion with *Hae*III and *Fok*I as recommended by the suppliers (Boehringer GmbH, Mannheim, FRG).

Taq-cycle sequencing of the PCR amplification products

Prior to sequence analysis, digestion of the PCR products derived from strains harboring both *slt-IIB* and *slt-IIB*-related genes was performed with *Fok*I, which selectively digests *slt-IIB*. The procedure was repeated with the undigested fragment consisting of *slt-IIB*-related genes, in order to eliminate small quantitiies of *slt-II* that had

not been digested by the first procedure. Gel slices with the undigested fragments were excised, the DNA being purified with the gene clean kit as described by the manufacturer (Dianova, Hamburg, FRG). A total of 1 μg of the double-stranded PCR product was subjected to Taq-cycle sequencing reactions using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Darmstadt, FRG). Briefly, 9.5 μl terminator premix, template DNA (1 μg) and 3.2 pmol of primer GK5 or GK6 were mixed in a 0.6-ml reaction tube and filled up with distilled water to make a final volume of 20 μl. The tubes were placed in a thermal cycler preheated to 96°C and subjected to 25 cycles with the following parameters: 96°C for 15 s, 52°C for 15 s and 60°C for 4 min. The cycle sequencing products were extracted with phenol/chloroform and ethanol-precipitated. The resulting DNA pellets were dissolved in 4 μl of a mixture of formamide and 50 mM EDTA pH 8.0 in the ratio 5:1. Separation of sequencing products was performed on 7% denaturing polyacrylamide gels in an automatic sequencer (Model 373 A, Applied Biosystems, Darmstadt, FRG). Nucleotide sequencing was carried out in triplicate and analyzed with the DNAsis program, version 2.0 from Pharmacia LKB.

Cytotoxicity and neutralisation assays

Cytotoxicity assays were performed with Vero cells. Neutralisation assays with anti-SLT-I and anti-SLT-II were carried out as described [18].

Results and Discussion

Fifteen *E. coli* O157 strains from six patients with hemorrhagic colitis and from nine patients with hemolytic–uremic syndrome were analyzed. These strains were selected because they showed cytotoxic activity to Vero cells that could not be completely neutralized by anti-SLT-I and/or anti-SLT-II antibodies. Therefore genotypic classification was performed. The findings of SLT-II subtyping performed with DNA probes and by PCR as well as restriction fragment polymorphism of the PCR products are presented in Table 1. Six strains harbored only *slt-II*-related genes, seven strains possessed *slt-II*-related genes together with *slt-II*, and two strains had *slt-II*-related genes plus *slt-I*.

Table 1

Disease association and *slt* genotypes of the 15 *E. coli* O157 isolates investigated in this study

Strain	Disease strain is associated with	Origin/Reference	Year of isolation	Genotypic classification of SLTs performed by DNA-hybridization, RFLP and nucleotide sequence analysis of *slt-IIB*-related genes
4821/87	HC	Freiburg, FRG (this study)	1987	*slt-IIc*
7513/91	HUS	Münster, FRG [19]	1991	*slt-IIc*
6983/92	HUS	Hannover, FRG [19]	1992	*slt-IIc*
2406/92	HUS	Erlangen, FRG [19]	1992	*slt-IIc*
3658/93	HUS	Hamburg, FRG [19]	1993	*slt-IIc*
A9123-1	HC	USA (CDC)	1982	*slt-IIc*
2780/88	HC	Hamburg, FRG (this study)	1988	*slt-I, slt-IIc*
5291/92	HUS	Marburg, FRG [19]	1992	*slt-I, slt-IIc*
1249/87	HUS	Oldenburg, FRG [19]	1987	*slt-II, slt-IIc*
3978/91	HUS	Göttingen, FRG [19]	1991	*slt-II, slt-IIc*
5130/91	HUS	Heidelberg, FRG [19]	1991	*slt-II, slt-IIc*
5650/91	HUS	München, FRG [19]	1991	*slt-II, slt-IIc*
6548/91	HC	Rostock, FRG (this study)	1991	*slt-II, slt-IIc*
4248/89	HC	Bremen, FRG (this study)	1989	*slt-II, slt-IIc*
ED 39	HC	Rome, Italy (this study)	1992	*slt-II, slt-IIc*

HC, hemorrhagic colitis; HUS, hemolytic-uremic syndrome; RFLP, restriction fragment length polymorphism.

In strains carrying *slt-II*-related genes alone or in combination with *slt-I*, the PCR fragments were directly subjected to nucleotide sequence analysis. Direct sequencing was not possible with the seven strains possessing both *slt-II* and *slt-II*-related genes, since the PCR products contained both genes. In order to perform sequence analysis of these *slt-II*-related genes, the PCR products were first subjected to restriction enzyme digestion with *Fok*I, which selectively digested *slt-IIB* (Fig. 1, lane 5). With the strains possessing *slt-II* and *slt-II*-related genes, this resulted in an undigested 270-bp fragment consisting of pure *slt-II*-related genes (Fig. 1, lanes 7 and 8). Interestingly, comparison of the nucleotide sequences revealed a 100% homology of all 15 analyzed *slt-IIB*-related toxin genes. In addition, the nucleotide sequence of *slt-IIB*-related toxin genes were identical to *slt-IIcB*.

There is growing interest on variants of SLT-II, which were frequently detected among clinical *E. coli* O157 isolates. All eight strains analyzed by Tyler et al. [13] and 109 of 176 *E. coli* O157 strains studied by Thomas et al. [20] were shown to possess *slt-II*-related genes alone or in combination with *slt-I* or *slt-II*. In a previous study from our laboratory SLT-II-related toxins were identified in 17 of 38 *E. coli* strains from patients with HUS [18]. However, the precise SLT genotype of the variants has not been determined. Therefore, in this study nucleotide sequence analysis of the B-subunit genes was performed. The results demonstrated that the nucleotide sequences were identical to those published for *slt-IIcB* [6]. The predicted amino acid sequences of SLT-IIc are identical to those described for SLT-IIvha and SLT-IIvhc. However, SLT-IIc, SLT-IIvha and SLT-IIvhc differ slightly in their A-subunits. Since the A-subunit genes were not sequenced in the present study, there could be differences among the A-subunits of the SLT-II-related toxins analyzed here. Presently, according to the new nomenclature for SLTs, the above-mentioned toxins are collectively designated as

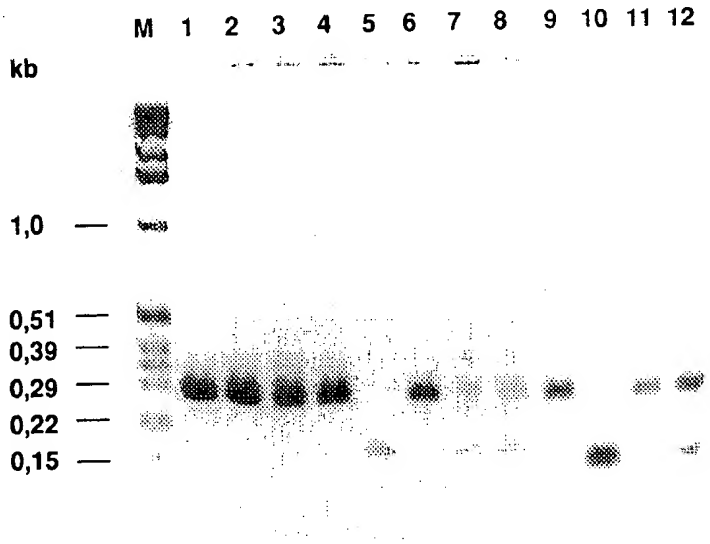

Fig. 1. PCR amplification products derived by amplification with primers GK5-GK6 and restriction enzyme analysis with *Fok*I and *Hae*III to differentiate between *slt-IIB* and *slt-IIB*-related gene sequences, prior to nucleotide sequence analysis. PCR product of *E. coli* C600 (933W) (*slt-II*) undigested (lane 1), digested with *Fok*I (lane 5) and *Hae*III (lane 9). The PCR fragment of the *slt-IIc* harboring *E. coli* strain 4821/87 is shown in lane 2, digestions of this fragment with *Fok*I and *Hae*III are depicted in lanes 6 and 10, respectively. In lanes 3 and 4 PCR products of *E. coli* strains 3978/91 and 5130/91 possessing both *slt-IIB* and *slt-IIB*-related genes are shown. Digestions of these fragments with *Fok*I are shown in lanes 7 and 8, with *Hae*III in lanes 11 and 12. M indicates the DNA molecular mass marker V (Boehringer GmbH, Mannheim, FRG).

SLT-IIc [8]. In their B-subunits, SLT-IIc toxins contain an uncharged asparagine residue at position 16 whereas SLT-II have a negatively charged aspartic acid residue at this location. It has been speculated that this amino acid exchange is responsible for the lower cytotoxic activity of SLT-IIc toxins [7,21]. This may be a consequence of the lower affinity of binding to the glycolipid receptor Gb3 [7,21,22].

Although significant heterogeneity has been reported among variants of SLT-II in *E. coli* not belonging to serogroup O157, our data indicate that this heterogeneity does not seem to exist between the SLT-II-related toxins of *E. coli* O157. This may be explained by a high clonal relationship of *E. coli* O157 strains as indicated by multilocus enzyme electrophoresis and DNA fingerprinting [23,24]. In addition, a transfer of *slt-II*-related genes from non-O157 strains to *E. coli* O157 does not seem to occur. Unlike SLT-I and SLT-II, variants of SLT-II are not encoded by bacteriophages [5–7].

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Isolation and Characterization of a β-D-Glucuronidase-Producing Strain of *Escherichia coli* Serotype O157:H7 in the United States

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A phenotypic variant of *Escherichia coli* serotype O157:H7 (G5101) was isolated from a patient with bloody diarrhea. Strain G5101 does not ferment sorbitol but is β-D-glucuronidase and urease positive. Serotyping and colony hybridization using a serotype-specific DNA probe confirmed that the isolate was O157:H7. G5101 produces Shiga-like toxins I and II and contains an *eae* gene that is highly conserved in the O157:H7 serotype. This strain would have been missed by laboratories that screen for the sorbitol-negative, β-D-glucuronidase-negative phenotype in isolating *E. coli* O157:H7 from clinical and food specimens.

Escherichia coli of serotype O157:H7 was first recognized as a cause of bloody diarrhea in humans in 1982, when two outbreaks in Michigan and Oregon were traced to ground beef contaminated with this organism (10). Isolates of O157:H7 and an occasional nonmotile variant belong to a pathogenic group known as enterohemorrhagic *E. coli* (EHEC). The pathogenicity of EHEC appears to be associated with a number of virulence factors that include attaching and effacing factors and the production of several cytotoxins. The cytotoxins (Shiga-like toxin I [SLT-I], SLT-II, and variants of SLT-II) are collectively known as SLTs because SLT-I is almost identical to the Shiga toxin of *Shigella dysenteriae* type 1. Although other *E. coli* serotypes share these virulence factors and have also been associated with human illness (4), *E. coli* O157:H7 remains the most frequent cause of hemorrhagic colitis, which may progress to the life-threatening hemolytic-uremic syndrome (HUS).

Isolates of serotype O157:H7 and the nonmotile variant group cluster in a few closely related enzyme types by multilocus enzyme electrophoresis and are more distantly related to other *E. coli* isolates (13). This high degree of genetic homogeneity has provided some unique metabolic phenotypes, which have facilitated the isolation and identification of serotype O157:H7 from food, clinical, and environmental specimens. Unlike other *E. coli* isolates, *E. coli* O157:H7 isolates do not ferment sorbitol in 24 h; hence, differential selection on MacConkey agar containing sorbitol has been very effective in isolating this pathogen from bloody stool specimens (4). Serotype O157:H7 also does not exhibit β-glucuronidase (GUD) activity (11); therefore, many laboratories, particularly those involved in food analysis, further screen sorbitol-negative colonies for GUD (7, 12). GUD assays are done easily by incorporating commercially available fluorogenic or colorimetric substrates in routine culture media. In their analysis of ground beef samples, Okrend et al. (7) reported that further screening of sorbitol-negative colonies for GUD reduced the number of false-positive identifications by 36% from that found by selection solely on the basis of the sorbitol phenotype.

We report the first isolation in the United States of an atypical O157:H7 isolate that does not ferment sorbitol but produces an active GUD. This strain, designated G5101, was isolated in April 1994 from a 20-year-old female student at the University of Washington, who had bloody diarrhea. She was not hospitalized. The source of the O157:H7 isolate that caused the gastrointestinal illness was not determined.

Serotyping with anti-O157 and anti-H7 sera identified strain G5101 as O157:H7. Furthermore, colony hybridization analysis using a serotype-specific DNA probe (PF-27) also confirmed G5101 as O157:H7 serotype (Fig. 1). PF-27 is an oligonucleotide probe, specific for a unique base substitution in the allele of the *uidA* gene in O157:H7 isolates (2). However, a previous study showed that the probe also detected phenotypic variants of O157 serotype that were nonmotile, fermented sorbitol, and like our G5101 strain, exhibited GUD activity (2). These atypical pathogenic O157 strains were isolated from HUS patients in Germany and obtained from H. Karch (5).

PCR analysis of strain G5101 showed that it carried virulence genes typically present in O157:H7 strains. Amplification of the SLT genes using primers and conditions described previously yielded amplicons with predicted sizes for SLT-I and SLT-II of 475 and 863 bp, respectively (8). The production of both toxins was confirmed with a commercially available enzyme immunoassay (Meridian Diagnostics, Cincinnati, Ohio). Amplification of the *eaeA* gene using primers from the central region of the gene, which is conserved between EHEC and enteropathogenic strains of *E. coli* (forward primer from nucleotides 2241 to 2263 and reverse primer from nucleotides 3350 to 3328 of the sequence reported by Jerse et al. [6]), and conditions described above for the SLT genes yielded an amplicon of the predicted size of 1,110 bp.

Biochemical characterization showed that, in addition to having an atypical GUD phenotype, G5101 was also urease positive in 48 h. Although *E. coli* strains are usually negative

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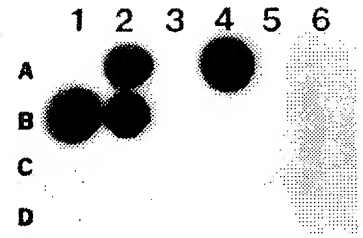

FIG. 1. Autoradiogram of colony hybridization of *E. coli* O157:H7-specific PF-27 probe to *E. coli* and other enteric bacteria. The probe assay was done as previously described (2). The serotype of each isolate is shown in parenthesis. The positive controls are isolates EC177 (O157:H7) (A2), ATCC 35150 (O157:H7) (B1), and ATCC 43888 (O157:H7) (B2). The negative controls are isolates FDA207 (*Escherichia hermannii*) (B4) and FDA400 (H10407) (B5). The samples are isolates G5101 (O157:H7) (A4), ATCC 29026 (*S. dysenteriae*) (C1), ATCC 35401 (O78:H11) (C2), ATCC 43886 (O25:K98:NM) (C3), ATCC 43887 (O111) (C4), ATCC 43893 (O124:NM) (C5), and ATCC 43896 (O78:K80:H12) (C6). All other positions on the colony filter are blanks.

for urease (1), 24 cases of hemorrhagic colitis were caused by SLT-producing, urease-positive *E. coli* O157 in Canada through 1991 (9).

There is increasing evidence suggesting that phenotypic variations exist among the isolates within *E. coli* O157:H7. In Germany, Gunzer et al. (5) found that of 44 SLT-II-producing *E. coli* O157 strains isolated from patients with diarrhea or HUS, 17 fermented sorbitol and were GUD positive. Phenotypic variants of *E. coli* O157 have also been isolated in other parts of central Europe (3). Strain G5101 is the first phenotypic variant of the O157:H7 serotype isolated in the United States. Clinical and food microbiologists should be aware of the emergence of these phenotypic variants and be cognizant that these strains may not be identified by routine culture methods or by biochemical tests used to characterize serotype O157:H7.

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NOTES

DNA Fingerprinting of *Escherichia coli* O157:H7 Strains by Pulsed-Field Gel Electrophoresis

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Pulsed-field gel electrophoresis of genomic DNA was carried out on *Escherichia coli* O157:H7 strains from different geographic locations to determine its value in an epidemiological survey of O157 infections. Pulsed-field gel electrophoresis of *Xba*I-digested DNA fragments clearly separated *E. coli* O157:H7 strains from nontoxigenic *E. coli* O157:H19, O157:H43, and O157:H45 strains and from Shiga-like-toxin-producing *E. coli* strains of other serogroups. However, among the *E. coli* O157:H7 strains, the restriction patterns either were identical or differed only by a few fragment bands. In some cases, it was therefore impossible to distinguish among epidemiologically unrelated strains. Hybridization experiments with a DNA probe complementary to Shiga-like toxin II sequences revealed that the Shiga-like toxin II genes were located on DNA fragments of different lengths. Our data show that for a single highly conserved clone, such as *E. coli* O157:H7, other typing techniques may need to be performed in addition to DNA fingerprinting in epidemiological surveys.

Shiga-like toxin (SLT)-producing *Escherichia coli* is now well recognized as a human pathogen causing serious illness in the form of hemorrhagic colitis and hemolytic-uremic syndrome (8, 13). The prototype SLT producers are strains of serotype O157:H7. The pathogenic mechanisms utilized by these pathogens in the course of infection are poorly characterized but seem to involve the production of SLTs (7). One of these toxins, Shiga-like toxin II (SLT-II), expressed either alone or in concert with Shiga-like toxin I (SLT-I), seems to be produced by most *E. coli* O157:H7 strains associated with human disease (10, 14, 15). The epidemiology of *E. coli* O157:H7 infections is poorly understood. Phenotypic and genotypic schemes have been developed to assist in epidemiological investigations. These include typing of the strains by phages and by plasmid and toxin profiles (1, 10, 14, 15). In addition, multilocus enzyme electrophoresis has been performed (15), and the data indicate that North American *E. coli* O157:H7 strains of recent descent belong to one clone. In this study, we utilized pulsed-field gel electrophoresis (PFGE) to conduct molecular subtyping of *E. coli* O157:H7 strains from different geographic locations to determine whether this technique can differentiate epidemiologically independent strains.

(The data in this paper will appear in the M.S. thesis of H. Böhm [University of Würzburg, Germany].)

Thirty-six *E. coli* O157:H7 isolates from diverse geographic locations were used in this study (Table 1). In addition, four isolates of serogroup O157 that were either nonmotile or expressed the H19, H43, or H45 antigen were analyzed. These isolates were described previously (2). We further investigated seven SLT-producing *E. coli* isolates belonging to serotypes O111:H$^-$, O26:H11, O22:H8, and O55:H6; one enteroinvasive *E. coli* isolate; and one uropathogenic *E. coli* isolate. The reference strains for SLT-I and SLT-II were *E. coli* C600(H19J) and *E. coli* C600 (933W), respectively. The bacterial strains were grown overnight in double yeast-tryptone medium at 37°C with constant shaking. Genomic DNA was isolated by a modified version of the method of Grothues and Tümmler (4). In brief, for PFGE, 1 ml of an overnight culture was used to inoculate an exponential culture, which was grown in double yeast-tryptone medium to an optical density at 600 nm of about 1.0. Ten milliliters of this cell suspension was pelleted by centrifugation, and the pellets were washed three times with 75 mM NaCl–25 mM EDTA (pH 7.4) (SE) and resuspended in 1 ml of SE. Finally, 125 μl of this suspension was mixed with 875 μl of SE and 1 ml of 2% genetic technical grade agarose (Rotaphor grade; Biometra, Göttingen, Germany) in buffer consisting of 10 mM Tris, 10 mM $MgCl_2$, and 0.1 mM EDTA (pH 7.5). The mixture was carefully dispensed into aluminium molds (140 by 7 by 2 mm). After 10 min at 4°C, the solidified preparations were cut into plugs of an appropriate size. Cell lysis was carried out overnight at 56°C with vigorous shaking in 5 ml of 50 mM Tris–50 mM EDTA–1% *N*-lauroylsarcosine, 2 mg of proteinase K per ml (pH 8.0). On the following day, the agarose plugs were washed with 10 mM Tris–5 mM EDTA (pH 7.5) (TE) and stored in 50 mM EDTA. Digestion of the agarose-embedded DNA was carried out with *Xba*I, *Pac*I, and *Sfi*I (New England Biolabs, Beverly, Mass.) and with *Not*I (LKB Pharmacia, Uppsala, Sweden) in accordance with the manufacturers' instructions: 10 U of *Xba*I at 37°C for 4 h, 10 U of *Not*I and 10 U of *Pac*I at 37°C for 16 h, and 10 U of *Sfi*I at 50°C for 16 h. The reaction was stopped by the addition of 20 μl of 0.25% (wt/vol) bromphenol blue–0.1% sodium dodecyl sulfate (wt/vol) in TE. PFGE was performed with a Rotaphor R22 apparatus (Biometra) and 1% genetic technical grade agarose–Tris-borate buffer gels in accordance with the manufacturer's instructions. After a 26-h run at a constant voltage of 200 V, pulse times of 5 to 50 s with linear ramping, an electrical field angle of 120°C, and a temperature of 13°C, the

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TABLE 1. Sources and phenotypic and genotypic characteristics of *E. coli* strains used in this study

Strain	Serotype	Clinical disease; source[a]	Numeric difference of fragments compared with strain EDL 933	No. of fragments of sizes different from those in strain EDL 933	Groups of identical RFP[b]
EDL 933	O157:H7	Meat; CDC	0	0	A
EDL 932	O157:H7	HC; CDC	2	2	
EDL 931	O157:H7	HC; CDC	1	1	B
A 8993 CS2	O157:H7	HC; CDC	0	0	A
A 9167-1	O157:H7	HC; CDC	0	8	
A 9047 CS1	O157:H7	HC; CDC	0	0	A
A 8979 B1	O157:H7	HC; CDC	1	1	
A 9123	O157:H7	HC; CDC	1	3	
HUS CL8	O157:H7	HUS; CND	1	1	
HUS CL40	O157:H7	HUS; CND	2	2	C
H 512/87	O157:H7	HC; D	1	3	
H 1071/87	O157:H7	HUS; D	1	7	
H 709/90	O157:H7	HC; D	1	7	
4220/87	O157:H7	HC; D	0	0	A
H636/87	O157:H7	HUS; D	1	1	B
H 660/88	O157:H7	EC; D	0	6	
H 845/88	O157:H7	HC; D	0	6	
H 356/88	O157:H7	HC; D	2	4	
H 637/87	O157:H7	HC; D	2	4	
7279/87	O157:H7	EC; D	0	2	
4821/87	O157:H7	EC; D	0	0	A
660/88 Wü	O157:H7	HUS; D	0	6	
3978/89 Wü	O157:H7	HUS; D	2	6	
3268/90 Wü	O157:H7	HUS; D	2	2	C
3269/90 Wü	O157:H7	HUS; D	3	5	
0705/91 Wü	O157:H7	HUS; D	2	2	C
0729/91 Wü	O157:H7	HUS; D	1	3	
1658/91 Wü	O157:H7	HUS; D	0	2	
3850/91 Wü	O157:H7	HUS; D	2	6	
4740/91 Wü	O157:H7	HUS; D	0	2	
4739/91 Wü	O157:H7	HUS; D	1	7	
5769/87	O157:H7	HUS; D	1	1	B
9336/89	O157:H7	HUS; D	1	5	
4242/87	O157:H7	HC; D	1	7	
631/88	O157:H7	EC; D	4	6	
637/87	O157:H7	HC; D	2	4	
629/91	O157;H−	HUS; D	5	17	
241-36/88	O157:H43	EC; D	5	17	
904/90	0157:H45	EC; D	1	19	
693/91	O157:H19	EC; D	5	17	
3278/89	O111:H−	HC; D	2	28	
HUS 2/86	O111:H−	HUS; D	2	24	
4503/87	O26:H11	EC; D	3	19	
138-46/89	O22:H8	Milk; D	1	13	D
138/89	O22:H8	HUS; D	1	13	D
700 36/85	O55:H6	EC; D	1	19	E
701 36/85	O55:H6	EC; D	1	19	E
76/5	O143:H−	DYS	1	17	
WF 96	O7:H6	UTI; GB	3	23	

[a] CDC, Centers for Disease Control, Atlanta, Ga.; CND, Canada; D, Germany; DYS, dysentery; EC, enterocolitis; GB, Great Britain; HC, hemorrhagic colitis; HUS, hemolytic-uremic syndrome; UTI, urinary tract infection.

[b] RFP, restriction fragment pattern. Strains with identical RFPs form a group. Different groups are designated by different letters (A through E). Absence of a letter indicates a unique RFP not shared with other strains.

gels were stained with ethidium bromide. A lambda ladder PFGE marker was purchased from New England Biolabs. To determine the relationship among the different strains, we first compared the number and size of the fragments. All strains were compared with each other. Vacuum blotting was carried out on a VacuGene apparatus (LKB Pharmacia) in accordance with the manufacturer's instructions but with slight modifications. A 230-bp fragment of the SLT-II gene was amplified with primer pair MK1-MK2 (6) and DNA from *E. coli* C600 strain 933W. The amplified fragment was labeled with digoxigenin (Boehringer, Mannheim, Germany). Labeling of the probe, hybridization, and detection were performed in accordance with the manufacturer's instructions. For the detection of the lambda ladder, digoxigenin-labeled lambda DNA (LKB Pharmacia) was used.

Chromosomal DNAs from five selected strains were initially digested with *Not*I, *Sfi*I, *Pac*I, and *Xba*I. *Xba*I appeared to give the best resolution and was used for all study strains listed in Table 1. Figure 1A shows the *Xba*I patterns of 17 *E. coli* strains, 12 of which were *E. coli* O157:H7 strains (lanes 1 to 12). By comparison of restriction fragment patterns, all *E. coli* O157:H7 strains were characterized by common fragments of about 440 and 350 kb. In 34 strains, five identical fragments in the range of 220 to 300 kb were observed, except in strains 7279/87 and A 9167/1, which had six identical fragments (Fig. 1A, lanes 1 and 7). All strains were compared with each other, and the number of fragments not identical to those in strain EDL 933 is shown in Table 1. No more than eight nonidentical fragments were present. In four strains, the number and size of the fragments were identical. The patterns of the chromosomal DNAs of strains identical to strain EDL 933 are shown in Fig. 1A, lanes 2, 3, 5, and 8. However, at least two of these identical strains were not related epidemiologically. For example, North American meat strain EDL 933 was identical to German strains 4821/87 and 4220/87 (Table 1). Moreover, strain EDL 933 was also highly related to Canadian hemolytic-uremic syndrome strain HUS CL8 (Table 1 and Fig. 1A, lane 9), differing in only a single fragment. In addition, *E. coli* O157:H7 strain EDL 931 was identical to German hemolytic-uremic syndrome strain H636/87. These findings of genotypic identity within O157:H7 strains were verified by the use of *Not*I for digestion of the DNA (data not shown). When strain EDL 933 or the other O157:H7 strains were compared with *E. coli* O157:H15, O157:H19, and O157:H43 strains or with SLT-producing *E. coli* strains of other serogroups, many fragments were found not identical (Table 1).

*Xba*I fragments separated by PFGE and hybridized with the probe derived from the SLT-II gene revealed specific binding to the SLT-II reference strain (Fig. 1B, lane 17) but not to the SLT-I reference strain (Fig. 1B, lane 16). SLT-I-producing North American *E. coli* O157:H7 strain A 9123, originating from a patient with hemorrhagic colitis, did not hybridize with the probe either (Fig. 1B, lane 6). All the other clinical isolates producing SLT-II or toxins closely related to SLT-II hybridized, but with fragments of different sizes, i.e., 60 kb (Fig. 1B, lane 1), 210 kb (Fig. 1B, lane 13), 245 kb (Fig. 1B, lane 4), 530 kb (Fig. 1B, lanes 2, 3, 5, 8, 9, and 11), and 605 kb (Fig. 1B, lane 12). Strain A 9167-1 had two fragments of 110 and 130 kb that hybridized (Fig. 1B, lane 7). Because of the fact that the SLT genes sequenced to date contain no *Xba*I site, these two bands may well represent duplicate copies of the SLT-II gene or closely related toxin genes.

The need for a single and reliable method of comparing *E. coli* O157:H7 strains is evident because these pathogens are increasingly implicated in human diseases. In many cases, a typing scheme is warranted to identify the vectors responsible for the transmission of these organisms, be it through foodstuffs or through humans. In the case of other bacterial

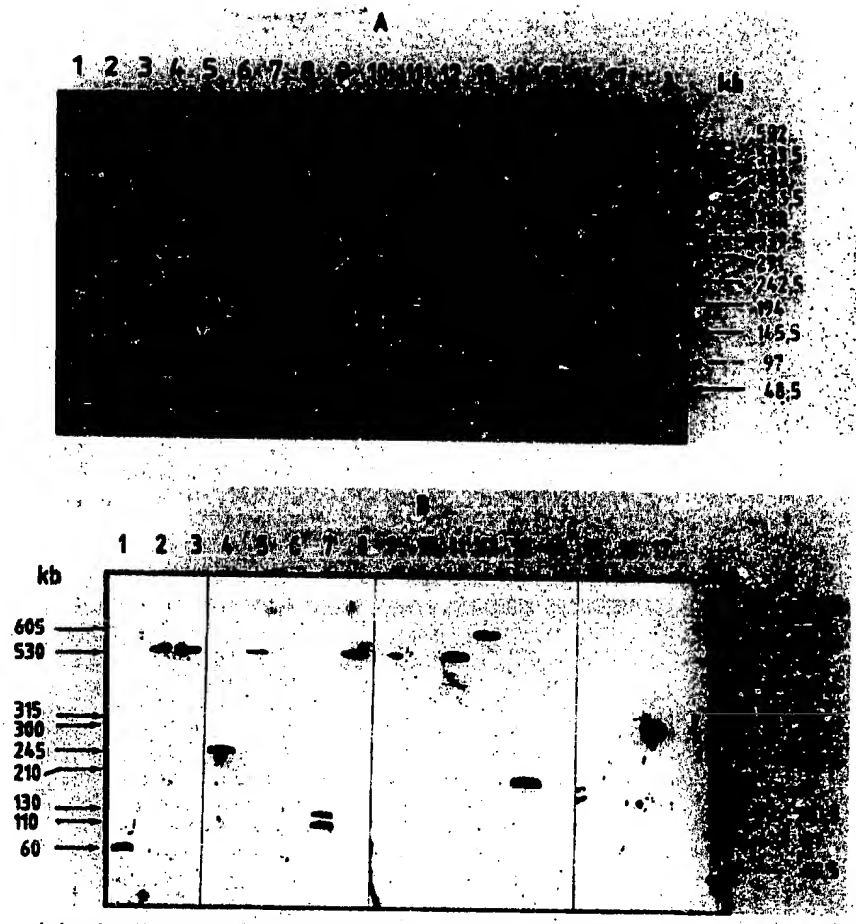

FIG. 1. (A) Agarose gel showing *Xba*I digestion patterns of *E. coli* O157:H7 strains (lanes 1 to 12). Strains 7279/87, 4220/87, and 4821/87 (lanes 1 to 3) were from Germany; strains A 8979 B1, A 8993 CS2, A 9123, A 9167-1, A 9047 CS1, EDL 933, and EDL 932 were from North America (lanes 4 to 8 and 11 to 12); and strains HUS CL8 and HUS CL40 were from Canada (lanes 9 and 10). Lane 13, *E. coli* O157:H− strain 629/91; lane 14, *E. coli* O157:H43 strain 241-36/88; lane 15, *E. coli* C600; lane 16, *E. coli* C600(933J); lane 17, *E. coli* C600(933W). Lambda DNA concatemers were used as DNA size markers. (B) Southern hybridization of the *Xba*I-cleaved genomic DNA shown in panel A with a DNA probe complementary to SLT-II sequences.

pathogens, such as *Pseudomonas* spp. (4), *Legionella* spp. (12), staphylococci (5), enterococci (9), or uropathogenic *E. coli* (11), PFGE was used successfully in comparative chromosomal DNA analyses for epidemiological purposes. In this study, PFGE was used to discriminate among strains of *E. coli* O157:H7 from different geographic areas. The *E. coli* O157:H7 restriction fragment patterns were very typical for this serotype and enabled us to identify a clinical *E. coli* isolate as O157:H7 without knowledge of the O and H antigens. The similarities in PFGE patterns observed in the present study are not unexpected, because a population genetic analysis done by multilocus enzyme electrophoresis has revealed that O157:H7 strains from diverse sources in North America belong to a single bacterial clone (15). According to our results, this clone has also spread from North America to Germany or vice versa. However, Arbeit et al. (3) recently reported that PFGE could differentiate epidemiologically independent but evolutionarily related isolates that were indistinguishable by multilocus enzyme electrophoresis and restriction polymorphisms associated with rRNA operons. On the other hand, our data show the limitations of PFGE when highly related strains are analyzed. It is difficult to unequivocally prove by PFGE that a given strain from meat or patients is responsible for the spread of an outbreak. The minor variations in restriction fragments could be due to different sites of integration of the phages into the O157 chromosome. In addition to DNA fingerprinting, other typing techniques should be used in epidemiological studies.

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THANK YOU

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Prevalence of the *eae*A gene in verotoxigenic *Escherichia coli* strains from dairy cattle in Southwest Ontario

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SUMMARY

This study determined the prevalence of the *eae*A gene and its relationship to serotype and type of verotoxin produced in a collection of 432 verotoxigenic *Escherichia coli* (VTEC) obtained from the faeces of healthy cows and calves in a systematic random survey involving 80 dairy farms in Southwest Ontario. A PCR amplification procedure involving primer pairs which target the conserved central region of the O157:H7 *eae*A gene showed that 151 (35·2%) strains were positive for the *eae*A gene. All isolates (9-21 for each O group) of O groups 5, 26, 69, 84, 103, 111, 145 and 157 were positive, whereas all isolates (7-34 for each O group) of O groups 113, 132, and 153 and serotype O156:NM (38 isolates) were negative for *eae*A. Seventy-three percent of 130 isolates of *eae*A-positive serotypes produced VT1 only compared with 20% of 253 isolates of *eae*A-negative serotypes. We conclude that there is a strong association between certain O groups and the *eae*A gene, that serotypes of *eae*A-positive and *eae*A-negative VTEC implicated in human and cattle disease are present at high frequency in the faeces of healthy cattle, that VT1 is more frequently associated with *eae*A-positive than with *eae*A-negative serogroups, and that the *eae*A gene is more frequently found in VTEC from calves compared with VTEC from adult cattle.

INTRODUCTION

Verotoxigenic *Escherichia coli* (VTEC) are characterized by their ability to produce at least one cytotoxic protein that is active on Vero cells and is referred to as verotoxin (VT) or Shiga-like toxin (SLT) [1, 2]. VTEC have been associated with a wide spectrum of human diseases, including diarrhoea, haemorrhagic colitis (HC) and the haemolytic uremic syndrome (HUS) [3–7]. Those VTEC serotypes which are able to induce haemorrhagic colitis are called enterohaemorrhagic *E. coli* (EHEC). Only a few serotypes, including O157:H7, O157:NM, O26:H11 and O111:NM have been clearly demonstrated to be EHEC and many serotypes isolated from humans with disease are of uncertain status. Nonetheless it is useful to use the term EHEC for referring to disease-producing VTEC.

Serotype O157:H7 is the most prevalent serotype implicated in outbreaks of VTEC-mediated disease in humans [1, 5, 7, 8]. VTEC isolates of serotypes O5:NM, O8:H9, O26:H11 and O111:NM have been associated with natural disease in calves 2–8 weeks of

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age and have been used in experimental reproduction of dysentery in calves [9–12]. VTEC of serotypes O26:H11 and O111:NM are frequently implicated in diseases in humans as well as calves [8, 10, 13–15], whereas VTEC of serotype O5:NM are frequent in cattle and less frequent in humans and VTEC of serotype O8:H9 have not been reported from humans [16]. Host species specificity is most evident with serotype O157:H7 which causes disease in humans but not in cattle. Serotypes of VTEC implicated in human disease have all been recovered from the faeces of cattle [16, 17] and foods contaminated with bovine faeces appear to be important sources of VTEC associated with human diseases [15, 18–20].

Certain VTEC cause attaching and effacing (AE) lesions in the large intestine similar to those caused by enteropathogenic *E. coli* (EPEC) in the small intestine and both types of *E. coli* are referred to as 'attaching and effacing *E. coli*' (AEEC) [21–27]. In EPEC, the chromosomal gene *eae*A encodes a 94-kDa outer membrane protein (intimin) which is necessary for intimate attachment to epithelial cells *in vitro* and *in vivo* [26, 28, 29]. A gene probe derived from the EPEC *eae*A nucleotide sequence recognizes a similar locus in certain VTEC strains [29].

Recently, two groups have cloned and sequenced the *eae*A gene from VTEC strains and have compared the sequences for the *eae*A gene from VTEC and EPEC strains [30, 31]. They have shown striking homology (97%) in the N-terminal and central regions, with less homology (59%) at the C-terminal region, which determines receptor specificity and is probably responsible for colonization of different regions of the intestine by these types of *E. coli* [30, 31]. Intimin is thought to bind membrane receptors which anchor the polymerized actin lying immediately underneath the adherent bacterium [32]. Recently it has been reported that the product of the VTEC *eae*A gene is a 97 kDa surface-exposed protein, which appears to play a key role in pathogenesis of the AE lesion [33].

Serotypes of VTEC implicated in bovine disease produce the same verotoxins as those which cause human disease [2] and factors required for colonization of the intestine are likely to be critical in determining virulence and host species specificity. The EaeA protein (intimin) is one factor which appears to be involved in the intimate adherence of pathogenic VTEC to the intestinal mucosa. In the present study, we examined a collection of bovine VTEC obtained in a systematic random survey to determine the prevalence of the *eae*A gene and its association with serotype, type of VT produced, and previous implication in disease of humans and cattle.

MATERIALS AND METHODS

Sampling

Eighty dairy farms from 12 counties in southern Ontario were selected by a formal random sampling procedure. A single rectal swab was taken from all healthy calves less than 3 months of age and from a random sample of the healthy milking herd, consisting of 25% of the herd or a minimum of 10 cows. This plan resulted in samples from 592 calves and 886 cows. Faecal swabs were transported on ice to the laboratory. The faecal swab was added to 9 ml of MacConkey broth (Difco, Detroit, MI) and incubated overnight at 37 °C. Next morning 500 μl of MacConkey broth culture were added to 5 ml of brain heart infusion broth (BHI) (Difco) and incubated for 24 h. The BHI culture was centrifuged at 12000 *g* in a microfuge and the supernatant used in a Vero cell assay (VCA) for detection of VT [34].

Attempts were made to isolate individual VT-producing colonies from samples that were positive in the screening test of BHI culture supernatant. The MacConkey broth cultures were streaked onto MacConkey agar plates which were incubated at 37 °C overnight. Five individual colonies and a sweep of colonies from the initial streak area were then tested for VT production, using the VCA. When only the colony sweep was positive, further testing was done to identify positive colonies. The specificity of the cytotoxicity on Vero cells was confirmed by polymerase chain reaction (PCR) amplification to determine whether the isolates possessed the genes for VT1, VT2 or both VT1 and VT2 [35].

Polymerase chain reaction

Primers C1 (5′-TCGTCACAGTTGCAGGCCTGG-T-3′ [803-824]) and C2 (5′-CGAAGTCTTATCCGC-CGTAAAGT-3′ [1912-1890]) were used in the PCR reaction to amplify a 1·1 kg DNA fragment (base pair 803-1912). These primers, derived from the conserved central region of the *eae*A gene of *E. coli* O157:H7 [29], were prepared in the Health of Animals Laboratory, Guelph. A 1 ml volume of overnight culture of the bacterium in Brain Heart Infusion broth (Difco, Detroit, MI) was centrifuged and the pellet

washed in FA buffer (Difco, Detroit, MI) then suspended in 500 μml double distilled water. The bacterial suspension was boiled for 10 min then cooled on ice for 2 min. The supernatant was used as template DNA. Amplification reactions of 25 μl consisted of 1 mM 1X PCR buffer, 1·2 4 μl Taq polymerase (GeneAmp, Perkin Elmer Cetus, Norwalk, CT), 20 pmol. of each primer and 5 μl template DNA. Amplification was performed in a GeneAmp PCR system 9600 (Perkin Elmer Cetus) for 35 cycles as follows: 94 °C for 1 min, 55 °C for 1 min at 72 °C for 1·5 min. A negative control which contained all components of the reaction mixture, with the exception of template DNA was included in each set of samples tested. Two O26:H11 and two O157:H7 VTEC were used as positive controls. All samples were tested with the C1 and C2 primers. After amplification, 10 μl of the reaction mixture was analyzed by agarose gel electrophoresis.

Specificity of the amplification produce was confirmed by two methods. First, the amplification products from 30 isolates, selected at random, were digested with restriction endonucleases *Cla*I and *Ban*I, which cleave the 1109 bp product to yield fragments of 567 and 542 bp and 674 and 435 bp, respectively. The digested DNA was analyzed by agarose gel electrophoresis. Secondly, the 1109 bp product from the O157:H7 positive control strain was digoxigenin-PCR labelled (Boehringer Mannheim) and used as a probe in hybridization studies under stringent conditions according to the manufacturer's instructions to confirm the identity of the amplification products derived from the 30 isolates.

Serotyping

All isolates were serotypes at both the National Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control, Ottawa, and the Agriculture Canada Health of Animals Laboratory, Guelph.

RESULTS AND DISCUSSION

A total of 608 VTEC were isolated. These consisted of 422 unique isolates and 186 isolates which were second colonies picked from positive samples. This latter group served as internal controls and are not included in the data presented. The results with the second colony were always the same as with the first colony from the same animal.

PCR amplification indicated that 145 (34%) of the 422 VTEC isolates possessed the *eae*A gene (Tables 1, 2). For 26 serotypes, all isolates were positive for the *eae*A gene (Table 1). Some serotypes (section B) were represented by low numbers, but were recorded so that their identity may become a part of the literature on serotypes which occur at low frequency. Of the 145 *eae*A-positive isolates (Tables 1, 2), 21 could not be typed precisely (Table 2). Among the 277 *eae*A-negative isolates (Tables 2, 3), 108 could not be typed completely. They include 80 O untypable isolates (section C, Table 3), which belong to 14 H antigen types, with more than half of these being H21.

To our knowledge this is the first study in which the prevalence of the *eae*A gene has been examined in VTEC obtained in a randomly collected manner from

Table 1. *Serotypes of verotoxigenic* E. coli *characterized as* eae*A-positive**

Serotype	Number tested	VT1	VT2	VT1 and VT2
A				
O5:NM†	14 (1)‡	10 (1)	0	4
O26:H11	17 (1)	16 (1)	1	0
O69:H11	5	4	1	0
O84:NM	8	8	0	0
O98:H25	4 (2)	4 (2)	0	0
O103:H2	19 (3)	19 (3)	0	0
O111:H8	4	2	0	2
O111:NM	7 (1)	7 (1)	0	0
O145:NM	12 (4)	4	8 (4)	0
O156:H25	3	3	0	0
O157:H7	13 (4)	1	4 (2)	8 (2)
B				
O5:H11	1	1	0	0
O18:H11	1	1	0	0
O26:NM	1	1	0	0
O49:NM	1	0	1	0
O74:NM	1 (1)	1 (1)	0	0
O76:H25	1	1	0	0
O80:NM	2	0	2	0
O84:Hunty§	1	1	0	0
O98:NM	2	1	1	0
O103:NM	1	1	0	0
O118:H16	2	2	0	0
O118:NM	1	1	0	0
O119:H25	1	1	0	0
O145:H8	1	1	0	0
O172:NM	1	0	1	0
Total	124 (17)	91 (9)	19 (6)	14 (2)

* All isolates that were tested were positive for the *eae*A gene.

† NM, non-motile.

‡ Numbers in parentheses are numbers of isolates from cows.

§ unty, untypable.

Table 2. *Verotoxigenic* E. coli *characterized as* eae*A-variable*

Group of strains	Number tested	*eae*A +ve	VT1	VT2	VT1 and VT2
O119:NM*	6	1	6 (1)†	0	0
OR:NM‡	4 (1)	1	1 (1)	2 (1)	1
O?:H25§	11 (8)	1 (1)	4 (1) (4)	3 (1)	4 (3)
O?:NM	28 (8)	18 (6)	4 (1)	23 (16) (7)	1 (1) (1)
Total	49 (17)**	21 (7)	15 (4) (4)	28 (16) (9)	6 (1) (4)

* NM, non-motile.
† Number in parentheses refers to *eae*A-positive isolates.
‡ OR, rough isolates.
§ O?, O antigen not in international scheme.
** Bold number in parentheses refers to number of isolates from cows.

a cattle population. Beutin and colleagues [36] recently reported that none of 33 VTEC isolates from healthy cattle was positive for *eae*A, but the collection of isolates was small and had not been obtained in a randomized manner. Seventeen of the 33 isolates were untypable but those that were typed were all different from those listed in Table 1. Three isolates from sheep that were found to be *eae*A-positive were of serotype O119:H25 (see Table 1). Interestingly, Beutin and colleagues [36] tested 52 O5:NM isolates (26 from sheep and 26 from goats) and found them all to be *eae*A-negative by the colony blot hybridization procedure. All 14 isolates of this serotype from healthy cattle that were tested by the PCR technique in the present study were positive.

The age of animal carrier may be related to prevalence of the *eae*A gene among VTEC isolates. It appears that the 33 isolates investigated by Beutin and colleagues [36] were from adult animals. In the present study, there were 24 *eae*A-positive isolates among 133 isolates (18%) from adult cattle, compared with 121 *eae*A-positive isolates among 289 (42%) isolates from calves (Table 4).

In contrast to the findings with VTEC from healthy cattle, Mainil and colleagues [37] found that 75% of 80 VTEC from calves with diarrhoea were *eae*A-positive by gene probe. The VTEC consisted of 70 isolates among 268 *E. coli* from Belgian calves with diarrhoea and 10 which had been received from researchers in the United States, Germany, the United Kingdom and Canada. Similarly, Barrett and colleagues [17] observed that 83% (29 of 35) of VTEC from calves with diarrhoea were *eae*A-positive, whereas only 49% (18 of 37) of VTEC from apparently healthy calves were *eae*A-positive. The high prevalence of *eae*A among VTEC from calves with diarrhoea [17, 37], the low prevalence of *eae*A among VTEC from healthy cattle [17, 36] and the higher prevalence of VTEC from healthy calves compared with healthy cows in this study suggest that *eae*A may be a factor in the colonization of the calf intestine by bovine VTEC.

Strains of serotype O157:H7 may affect the apparent association between *eae*A and pathogenicity for calves. In the study by Barrett and colleagues [17] 9 of the 18 *eae*A-positive isolates carried by healthy cattle were O157:H7 strains, which are associated with human but not bovine disease. In the present study, all O157:H7 strains (9 from healthy calves and 4 from healthy cows) were *eae*A positive (Table 1). It is interesting that isolates of this serotype constituted only 3% of the VTEC isolates, yet this serotype is the predominant serotype implicated in human disease. This finding suggests that this serotype may possess attributes other than *eae*A which favour its colonization of the human intestine and/or transmission among humans.

Among human VTEC, a high percentage of strains associated with disease carry the *eae*A gene [38, 39]. An exception was the finding by Wilshaw and colleagues [40] that there were only 13 *eae*A-positive isolates among 45 VTEC strains of human origin, that represented 17 O serogroups and were mostly from cases of HUS and HC. These results undoubtedly reflect the fact that the authors did not include strains of O groups 157 and 26 in their study, because it is well established that these possess the *eae*A gene.

Except for O groups 76 and 156, presence or absence of the *eae*A gene was related to O group. Thus, within each of Tables 1 and 3 it is common to find one O group with several H antigens or without the H antigen. There was a clear association of the

*eae*A gene with certain serotypes, including O5:NM, O26:H11, O69:H11, O103:H2, O111:NM, O145:NM and O157:H7 (Table 1), which have all been associated with disease in calves or humans. Other researchers [17, 29, 39, 40] have also found that the predominant O serogroups among typed *eae*A-positive strains were O5, O26, O111 and O157. Serotypes O80:NM and O84:NM have not been associated with disease, but all strains of these serotypes were positive for the *eae*A gene.

For 49 bovine VTEC (Table 2) the link between serotype and the *eae*A gene could not be made because the strains could not be assigned to a serotype. Non-motile strains of the same O group may represent more than one H type which have lost the ability to express flagella and or O? strains may represent strains of a single H type with different O antigenic origins. Possibly, a combination of serotyping with biotyping may be a simple method for distinguishing among groups of strains of the same O serogroup. Electrophoretic typing, although less simple, may be even more valuable in making these distinctions.

Certain VTEC serogroups which are associated with HC and HUS in humans lack the *eae*A gene. These include O91:HNM, O113:H21, O117:H4, O153:H25. Possibly, another gene fulfils the function of the *eae*A gene for these serotypes. A recent report [41] that VTEC O113:H21 isolated from human diarrhoea can efface microvilli in rabbit cecum is consistent with this hypothesis. It is more difficult to assess the role of the *eae*A gene in virulence of bovine VTEC, because the reported serotypes of *eae*A-negative cattle pathogens (O?:H16, O?:H19, O?:H21) lack identified O antigens and their relationship to isolates with the same designation in this study is uncertain.

There appeared to be an association of the *eae*A gene with type of VT produced (Tables 1, 2, 4). Prevalence of VT1 and of *eae*A was higher in VTEC from calves compared with those from cows (Table 4). Among 124 strains of serotypes characterized as *eae*A-positive, 73% produced VT1 only (Table 1), whereas among the *eae*A-negative serotypes only 20% produced VT1 only (Table 3). If one considers the established calf pathogenic serotypes (O5:NM, O26:H11, O103:H2 and O111:NM), then 52 of 57 isolates (91%) produced VT1 (Table 1). This finding is consistent with the report by Wieler and colleagues [42] who showed an association of VT1 with diarrhoeic compared with healthy calves and with the study by Mainil and colleagues who reported that,

Table 3. *Serotypes of verotoxigenic* E. coli *characterized as* eae*A-negative**

Serotype	Number tested	VT1	VT2	VT1 and VT2
A				
O2:H29	3 (1)†	0	3 (1)	0
O8:H19	5 (5)	0	5 (5)	0
O22:H8	13 (12)	3 (3)	1 (1)	9 (8)
O38:H21	4 (4)	1 (1)	2 (2)	1 (1)
O113:H4	17 (2)	7	1	9 (2)
O113:H21	10 (6)	0	6 (3)	4 (3)
O113:NM‡	7	0	2	5
O116:H21	3 (3)	1 (1)	1 (1)	1 (1)
O132:NM	10 (1)	1	9 (1)	0
O153:H25	12 (11)	3 (3)	5 (4)	4 (4)
O153:H31	7	7	0	0
O156:NM	38 (4)	0	33 (3)	5 (1)
B				
O2:H5	1	1	0	0
O2:H27	1 (1)	0	1 (1)	0
O2:NM	2 (1)	1 (1)	1	0
O6:H34	2 (1)	0	2 (1)	0
O7:H4	1	0	1	0
O8:H35	1	0	1	0
O22:H2	1 (1)	0	1 (1)	0
O39:H49	2 (2)	1 (1)	0	1 (1)
O46:H38	2 (2)	2 (2)	0	0
O46:NM	2 (2)	1 (1)	0	1 (1)
O76:H19	1 (1)	1 (1)	0	0
O76:Hunty§	2	2	0	0
O85:NM	1	1	0	0
O88:H25	1 (1)	0	1 (1)	0
O91:H7	1 (1)	0	0	1 (1)
O112:H2	1 (1)	1 (1)	0	0
O113:H7	1 (1)	0	1 (1)	0
O115:H8	1 (1)	1 (1)	0	0
O115:H18	2 (1)	0	0	2 (1)
O117:H4	2	0	0	2
O117:NM	1	0	0	1
O121:H7	1 (1)	1 (1)	0	0
O136:H12	1 (1)	1 (1)	0	0
O136:NM	1	1	0	0
O139:H19	1 (1)	0	1 (1)	0
O146:H8	1 (1)	0	0	1 (1)
O156:H7	1 (1)	1 (1)	0	0
O156:H8	1	0	1	0
O163:H2	1 (1)	0	1 (1)	0
O163:H19	1 (1)	0	0	1 (1)
O163:NM	1 (1)	0	0	1 (1)
O171:H2	1	0	1	0
C				
O Untypable	80 (25)	10 (7)	50 (10)	18 (8)
Total	249 (99)	50 (26)	132 (38)	67 (35)

* All strains that were tested were negative for the *eae*A gene.

† Numbers in parentheses are numbers of isolates from cows.

‡ NM, non-motile.

§ unty, untypable.

Table 4. *Distribution of verotoxins and* eae*A gene in VTEC from cows and calves*

	Number tested	*eae*A +ve	VT+	VT1	VT2	VT1 and VT2
Cows	886	24 (18)	133 (15)*	39 (29)	53 (40)	41 (31)
Calves	592	121 (42)	289 (49)	117 (41)	126 (44)	46 (16)
Total	1478	145 (34)	422 (29)	156 (37)	179 (42)	87 (21)

* Numbers in parentheses indicate percentages.

among 60 *eae*A-positive bovine VTEC from diseased animals, 93% produced VT1 as the only VT [37]. This pattern is different from that for O145:NM and O157:H7 (Table 1), two important human pathogens implicated in outbreaks of disease. Further studies are in progress to characterize these isolates with respect to their interaction with bovine colon to determine whether it may be possible to differentiate bovine and human pathogenic VTEC.

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1. EPIDEMIOLOGY AND INFECTION, (1994 Jun) 112 (3) 449-61.
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THANK YOU

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Sequence heterogeneity of the *eae* gene and detection of verotoxin-producing *Escherichia coli* using serotype-specific primers

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SUMMARY

The distribution of the *Escherichia coli* attaching and effacing (*eae*) gene in strains of verotoxin-producing *E. coli* (VTEC) isolated from cattle and humans was studied. The majority of strains isolated from humans with bloody diarrhoea or HUS and cattle with severe diarrhoea were *eae* positive (82 and 83% respectively). In contrast, 59% of VTEC isolated from asymptomatic cattle were *eae* negative and of the remaining 41% that were *eae* positive, the majority were serotype O157.H7. The nucleotide sequence of the 3′ end of the *eae* gene of enteropathogenic *E. coli* (EPEC) of serotype O55.H7 was found to be almost identical to that of serotype O157.H7. Specific primers are described which detect the *eae* sequences of VTEC serotypes O157.H7, O157.H$^-$, and EPEC serotypes O55.H7 and O55.H$^-$. The nucleotide sequence of the 3′ end of the *eae* gene of serotype O111.H8 differed significantly from that of O157.H7. Primers were developed to specifically identify the *eae* sequences of VTEC serotypes O111.H$^-$ and O111.H8. We conclude that whereas the majority of VTEC associated with disease in cattle and humans possess the *eae* gene, the gene itself may not be necessary to produce haemorrhagic colitis and HUS. Sequence heterogeneity in the 3′ end of *eae* alleles of VTEC permits specific identification of subsets of these organisms.

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INTRODUCTION

Verotoxin-producing *Escherichia coli* (VTEC) cause a wide spectrum of clinical disease in humans including non-bloody diarrhoea, haemorrhagic colitis (HC), the haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopoenic purpura [1–4]. Serotype O157.H7, the prototype of enterohaemorrhagic *E. coli* (EHEC) [5], is the most common cause of HC and HUS in North America and Western Europe. This serotype is also the most frequent cause of outbreaks in daycare centres and other institutions [6–9]. Other VTEC strains of diverse serotypes including those causing diarrhoea and HUS in humans are frequently isolated from animal faeces, meat and dairy products [10–17]. Cattle have been implicated as the principal reservoir for VTEC [16, 18, 19].

Recognition of the specific types of VTEC which are important causes of human disease, in animal and food samples presently depends on serotyping. Other diagnostic methods have been developed on the basis of the virulence properties of EHEC such as the DNA probe pCVD419 which detects the 60 megadalton plasmid of EHEC strains [20] and probes which detect the genes encoding Verotoxin 1 and Verotoxin 2 [21]. However, in animal specimens, toxin production does not distinguish VTEC which are important causes of human disease from those which are rarely human pathogens [11, 14]. Clearly other methods of identifying specific serogroups of VTEC would be useful.

Most VTEC strains which cause disease in humans and cattle colonize the intestine in a characteristic manner known as attaching and effacing (AE) [22–24]. This lesion is characterized by intimate adherence, effacement of microvilli and polymerization of actin in the underlying cytoplasm. Accumulation of polymerized actin can be demonstrated *in vitro* by the fluorescent actin staining (FAS) test [25]. The *eae* (*E. coli* attaching effacing) gene from an enteropathogenic *E. coli* (EPEC) strain has been cloned [26]. This gene which encodes a 94 kDa outer membrane protein called intimin, has been shown to be necessary but not sufficient for formation of the AE lesion [27, 28]. We and others have cloned and sequenced a similar *eae* gene from an EHEC strain of serotype O157.H7 [29, 30]. Although the EPEC and EHEC *eae* genes are highly conserved at the 5′ and central regions, they differ considerably at the 3′ end. The aim of this study was to determine the distribution of *eae* alleles in serotypes of VTEC and to see if the variation in the nucleotide sequence in the 3′ end could be used to develop allele-specific DNA probes and PCR primer sets.

METHODS

Bacterial strains

As summarized in Table 1, 146 well characterized VTEC strains of multiple serotypes and different sources were selected, to determine the serotype distribution of the *eae* gene. This group included 38 human strains consisting of 25 strains from cases of HUS and 13 strains from cases of bloody diarrhoea (Dr M. Karmali). A further 35 human VTEC strains were isolated from human faecal cultures (for which there was no clinical information). The remaining VTEC strains comprised 37 strains from asymptomatic cattle (isolated during surveys of

Table 1. *Verotoxin-producing* E. coli *strains used in PCR and colony hybridizations*

VTEC	Isolates: Human No.	Isolates: Cattle: Diseased No.	Isolates: Cattle: Not diseased No.	Total No.	*eae* Gene* Based on C1–C2 probe No.
O?.H16, 19, 21	—	4	4	8	0
O5.H⁻	—	8	1	9	9
O26.H11	6	7	1	14	14
O103.H2	—	2	2	4	4
O111.H⁻	8	4	—	12	12
O111.H8	2	2	—	4	4
O111.H11	—	6	—	6	6
O113.H21	3	—	3	6	0
O115.H18	—	2	—	2	0
O117.H4	2	—	—	2	0
O118.H16, 30	1	1	—	2	2
O121.H19	2	—	—	2	2
O145.H⁻	1	—	3	4	2†
O153.H21, 25	—	—	2	2	0
O156.H⁻	—	—	4	4	0
O157.H7	41	—	9	50	50
O157.H⁻	5	—	—	5	5
VTEC‡	2	—	8	10	0

* Presence of *eae* gene based on hybridization studies using the highly conserved central fragment C1–C2.

† *Eae* gene detected in two cattle O145 strains.

‡ Other VTEC (single isolates) include: cattle strains of serotypes O1.H20, O2.H29, O6.H34, O22.H16, O40.H8, O43.H2, O126.H8, O136.H16 and human strains of serotypes O18.H7, O91.H21.

beef cattle, dairy cows and veal calves) and 36 strains from calves with severe diarrhoea (isolated from faecal samples of sick animals referred from provincial laboratories across Canada where routine diagnostic procedures failed to isolate other known enteric pathogens).

Sixty-three verotoxin (VT) negative strains that represented the classical human EPEC serotypes as defined by the World Health Organization (WHO) [31] were used to test the specificity of O111.H8 and O157.H7 *eae* allele specific probes and primers. In addition, seven VT-negative serogroup O157 human strains were tested. Other bacterial strains used as negative controls included 10 non-pathogenic human faecal *E. coli*, 10 *E. coli* strains isolated from urine, 2 enterotoxigenic *E. coli*, 2 enteroinvasive *E. coli*, 2 *Shigella* sp., 1 *Yersinia enterocolitica* and 1 *Helicobacter pylori*. Bacterial strains were stored in citrated glycerol at −70 °C and were subcultured on unsupplemented Luria agar plates [32] prior to use.

Primers and probes

Table 2 shows the primers and probes used in polymerase chain reaction (PCR) and hybridization experiments. Synthetic oligonucleotide primers C1, C2, A2, S1, S2, P1EH and P2EH were based on the EHEC *eae* gene sequence [29]. Primers

Table 2. *Primers and probes. All primers were based on the EHEC* eae *gene, serotype O157.H7 except P40, P20 and B1 which were based on the* eae *gene of serotype O111.H8*

	Sequence (5′–3′)	Sense (+) Antisense (−)	Location within *eae* gene (BP)	Size of amplified product (KB)
Primers				
C1	TCGTCACAGTTGCAGGCCTGGT	+	803	1·1
C2	CGAAGTCTTATCCGCCGTAAAGT	−	1912	
P1EH	AAGCGACTGAGGTCACT	+	2442	0·45
P2EH	ACGCTGCTCACTAGATGT	−	2917	
S1	GCAGTAGCAAATGGTAAGGAT	+	2213	0·80
S2	TCTACACAAACCGCAT	−	3010	
P40	ACGTTACTGGTGACTTA	+	2508	0·40
P20	TATTTTATCAGCTTCAGT	−	2917	
Probes				
A2	GACCTCTTGACATTGT	−	2520	
B1	GCTGCAATATGGTCAGT	+	2539	

P40, P20, and B1 were based on the O111.H8 *eae* gene sequence determined in this study. Oligonucleotides A2 and B1 were used as internal probes for Southern hybridizations. Primers were purchased from the DNA Synthesis Laboratory, Samuel Lunenfeld Research Institute, Toronto, Ontario.

Polymerase chain reaction

Several bacterial colonies were selected, resuspended in 50 μl 1 × PCR buffer (Perkin Elmer Cetus, Norwalk, CT) and boiled for 10 min prior to use as template DNA in PCR assays. Amplification reactions of 50 μl were set up using 1 mM deoxynucleotide triphosphates, 1 × PCR buffer, 1 unit *Taq* polymerase (GeneAmp, Perkin Elmer Cetus, Norwalk, CT), 20 pmol of each primer and 1 μl template DNA. Amplification was performed in a Thermal Cycler, Model No. 480 (Perkin Elmer Cetus) for 40 cycles as follows: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. A negative control which contained all components of the reaction mixture, with the exception of template DNA, was included in each set of samples tested. After amplification, 10 μl of the reaction mixture was analysed by agarose gel electrophoresis.

Hybridizations

The 1·1 kb *eae* probe C1–C2 was made by PCR amplification using strain CL8 (serotype O157.H7) as template and primers C1 and C2. The 0·45 kb probe, P1EH–P2EH, specific for O157 strains was similarly amplified using primers P1EH and P2EH. The 0·40 kb probe, P40–P20, specific for O111 strains was amplified using strain RC541 (serotype O111.H8) as template and primers P40 and P20. DNA fragments used as probes were labelled with [α^{32}P] dATP using the Random Primed DNA Labelling Kit (Boehringer Mannheim). Oligonucleotide probes were end-labelled with [γ^{32}P] ATP using T4 polynucleotide kinase [32]. Colony blot and Southern hybridizations were performed overnight at 42 °C in

6 × SSC (1 × SSC is 0·15 M sodium chloride and 0·015 M sodium citrate), 5 × Denhardt's Reagent, 0·5% sodium dodecyl sulfate (SDS), 50% formamide and 100 μg of denatured herring sperm DNA [32]. Colony blots hybridized with probes labelled by random priming were washed as follows: 5 min at room temperature in 2 × SSC and 0·5% SDS, 15 min at RT in 2 × SSC and 0·1% SDS, 30 min × 2 at 68 °C in 0·1 × SSC and 0·5% SDS, and at RT in 0·1 × SSC. The Southern blot hybridized with the end-labelled A2 oligonucleotide was washed twice in 6 × SSC and 0·2% SDS at RT for 1 min, twice at 30 °C for 2 min, and once in 2 × SSC and 0·2% SDS at 34 °C for 3 min. The blot hybridized with the B1 oligonucleotide was similarly washed but the final wash was at 37 °C for 3 min.

Sequencing

DNA for sequencing the 3′ regions of the *eae* genes of strain AB9483.91 (serotype O55.H7), strain CL8 (serotype O157.H7) and strain RC541 (serotype O111.H8) was made by PCR amplification using bacterial colonies as template, as described above, and primers S1 and S2 (Table 2). Specific PCR products (0·8 kb) were excised from 1% agarose gels and the DNA was extracted by freezing the gel fragments at −70 °C spinning them through 0·22 μm cellulose acetate filters (CoStar Spin-X, Cambridge, MA) followed by phenol extraction and ethanol precipitation [32]. Direct PCR sequencing was carried out using the dsDNA Cycle Sequencing System (BRL Life Technologies, Gaithersburg, MD). The 0·8 kb purified PCR fragments were also subcloned into the vector pAMP™1 (BRL Life Technologies, Gaithersburg, MD) and sequencing was performed using single stranded DNA as previously described [29]. Sequence analysis was performed using the Wisconsin Genetics Computer Group Sequence Analysis Software Package Version 7.1 [33]. The 3′ *eae* gene sequences of serotypes O55.H7 and O111.H8 have been submitted to the Genbank database.

Tests for verotoxin

All bacterial strains (except negative control isolates) were tested for verotoxin production by cytotoxicity assays on Vero cells as described previously [11]. The presence of VT genes was confirmed using VT specific oligonucleotide primers in PCR assays [34].

RESULTS

Serotype distribution of eae *gene*

The conserved central region of the O157.H7 *eae* gene was used to determine the serotype distribution of *eae* alleles in the 146 VTEC strains isolated from humans and cattle. For colony blot hybridization, a 1·1 kb *eae* probe C1–C2 was produced by labelling a fragment amplified by PCR from the conserved central region, using primers C1 and C2 and DNA from serotype O157.H7 strain CL8 as template. This amplicon contained the 1·1 kb *Stu*I and *Sal*I fragment and has 98% sequence similarity with the *eae* probe described by Jerse and colleagues [26]. The results are presented in Table 1. *Eae* probe positive strains included serotypes O5.H$^-$, O26.H11, O103.H2, O111.H$^-$, H8, H11, O118.H16, H30, O121.H19, O145.H$^-$, and O157.H7, H$^-$. The presence or absence of the *eae* gene in strains of a

given serotype was consistent, with the exception of those of serotype O145.H⁻ where 1/1 human and 1/3 cattle strains were *eae* negative. Other bacterial isolates (28 strains) used as controls were all *eae* probe negative.

Human strains

Clinical histories were available for 38 strains which were isolated from patients with bloody diarrhoea or HUS. Of these, *eae* positive strains were of serotype O26.H11 (4), O111.H⁻ (5), O111.H8 (1), O118.H30 (1), O121.H19 (2), O157.H⁻ (2), O157.H7 (16), whereas *eae* negative strains were of serotypes O18.H7 (1), O91.H21 (1), O113.H21 (3), O117.H4 (1), and O145.H⁻ (1). The number of isolates of each serotype is indicated in parentheses. No clinical history was available for the remaining 35 strains of human origin.

Cattle strains

A total of 36 VTEC strains were isolated from cattle which were being investigated for severe diarrhoea or HC. No other enteric pathogens were isolated from these animals. Of these, 30 isolates were *eae* positive; they belonged to serotypes O5.H⁻ (8), O26.H11 (7), O103.H2 (2), O111.H⁻ (4), O111.H8 (2), O111.H11 (6) and O118.H16 (1). Six, including 4 O-untypable strains and 2 O115.H18 strains, were *eae* negative.

Thirty-seven VTEC strains were isolated from asymptomatic cattle during the course of surveys of cattle faecal samples. *Eae* positive strains included serotypes O5.H⁻ (1), O26.H11 (1), O103.H2 (2), and O145.H⁻ (2) and O157.H7 (9). *Eae* negative strains isolated from asymptomatic cattle included serotypes O?.H21 (3), O113.H21 (3), O156.H⁻ (4) and single isolates of serotypes O?.H19, O1.H20, O2.H29, O6.H34, O22.H16, O40.H8, O43.H2, O126.H8, O136.H16, O145.H⁻, O153.H21, O153.H25.

Nucleotide sequence of the 3′ end of eae *alleles*

To design primers which would detect specific VTEC serotypes, attempts were made to amplify and determine the nucleotide sequence of the 3′ regions of the *eae* alleles of selected VTEC strains of serotypes O5.H⁻, O26.H11, O111.H8, O111.H11, O118.H30 and an EPEC strain of serotype O55.H7. Using primers S1 and S2, specific 0·8 kb PCR amplification products were obtained only from a bovine isolate serotype O111.H8, and an EPEC strain of serotype O55.H7. The products were sequenced (data not shown) and the sequences were compared to those of the EPEC and EHEC *eae* genes which have been reported. The nucleotide sequences have been deposited in Genbank under the accession numbers L08095 (O55.H7) and L06255 (O111.H8). The deduced amino acid sequences are presented in Figure 1. Although there was significant nucleotide sequence (82%) similarity in the 3′ end of the *eae* alleles of serotypes O111.H8 and O157.H7, multiple amino acid substitutions were predicted in the putative protein products. The deduced amino acid sequence of the carboxyl end of the *eae* protein of the O55.H7 strain was virtually identical to that of the O157.H7 strain, differing only at position 770, 772, 773 and 904 (Fig. 1). By comparison, the deduced amino acid sequence of the O157.H7 strain reported by Yu and Kaper [30] only differed from the O55.H7 sequence at position 904.

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O157.H7  VKVMKNGQPVNNQSVTFSTNFGMFNGKSQTQATTGND-GRATITLTSSSAGKATVSATVS  740
O111.H8  .....E....QGH..V.T.............NA.TGSD.....................  741
O55.H7   ..........................................-.................  743
O127.H6  .....GDK..S..E...T.TL.---KL.NSTEK.DTN-.Y.KV....TTP..SL...R..  737

O157.H7  DGAE-VKATEVTFFDELKIDNKVD-IIGNN-VK-RSMLPNIWLQYGQFKLKASGGDGTYS  796
O111.H8  G.N-D...P......G.........-.L.K.-.T--GD..............V...N....  796
O55.H7   ....-.....................-.....-.--.GE.....................  798
O127.H6  .V.VD...P..E..TT.T..DGNIE.V.TG-...---GK..TV......VN......N.K.T  794

O157.H7  WYSENTSIATVDAS-GKVTLNGKGSVVIKATSGDKQTVSYTIKAPSYMI-----K-VDKQ  849
O111.H8  .H....N.....E.-.....K...TA..NV..............N.....-----R-.GNK  849
O55.H7   ..........-.....-.....-.....-.....-.....-.....-----.-....  851
O127.H6  .R.A.PA..S....S.Q...KE..TTT.SVI.S.N..AT...AT.NSL.VPNMS.R.TYN  854

O157.H7  -AYYADAMSICKNL---LPSTQTVLSDIYDSWGAANKYSHYSSMNSITAWIKQTSSEQRS  905
O111.H8  -.S..N...F.G..---...S.....NV.N.R.P..G.D..R..Q....R.T..EADKI.  905
O55.H7   -.....------.....-----.....-----.....-----.....-----.....S.  907
O127.H6  D.VNT-----...FGGK...S.NE.ENVFKA.......EY:K.SQT.IS.VQ..AQDAK.  909

O157.H7  GVSSTYNLITQNPLPGVNVNTPNVYAVCVE*                              936
O111.H8  ...T..D......HKD.Q..A.........*                              936
O55.H7   ..........................                                   931
O127.H6  ..A...D.VK....NNIKASES.A..T..K*                              940
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Fig. 1. Alignment of the deduced amino acid sequences at the 3′ end of: EHEC *eae* O157.H7 sequence [29], VTEC *eae* O111.H8 sequence, VTEC *eae* O55.H7 sequence, EPEC *eae* O127.H6 sequence [26]. The O111.H8, O55.H7 and O127.H6 amino acid sequences are compared with O157.H7 (strain CL8). Only positions which differ from the O157.H7 sequence are shown; identical position are displayed as dots (...) and spaces are represented by dashes (---). Amino acids of the O111.H8 and O55.H7 sequences were assigned numbers based on the alignment with the O157.H7 sequence.

Allele specific eae *probes*

Based on differences between the O157.H7 and O111.H8 *eae* gene sequences, primers P1EH, P2EH and primers P40, P20 were selected. Using an EHEC O157.H7 strain (CL8) as template, primers P1EH–P2EH were designed to amplify by PCR a 0·45 kb probe specific for O157 strains. This probe hybridized only with VT-positive strains of serotypes O157.H$^-$ and O157.H7 but not with VT-negative and *eae* negative O157 strains of the following H serotypes: H$^-$ (1), H16 (3), H19 (1), H25 (1) and H42 (1) (data not shown). The probe hybridized with EPEC strains of serotypes O55.H$^-$ and O55.H7, but not with other *eae* positive O55 strains. The probe also did not hybridize with any other EPEC strains which hybridized to the C1–C2 probe from the conserved central region (data not shown, see below).

A 0·40 kb probe P40–P20 was generated by PCR amplification using the O111.H8 strain RC541 as template. This probe hybridized only with VT-positive O111.H$^-$ and O111.H8 strains but not with VT-positive O111.H11 nor with the VT-negative strains of serotypes O111.H$^-$ and O111.H2 which hybridized with

Table 3. *PCR amplification products derived from various VTEC and EPEC O-serogroups using specific 3′ end primer sets*

Serogroup	Verotoxin	Specific PCR amplification products with primers	
		P1EH–P2EH*	P40–P20*
O5	+	0/9	0/9
O26	+	0/14	0/14
O55	−	11/16†	0/16
O103	+	0/4	0/4
O111‡	+	0/22	16/22
	−	0/6	0/6
O118	+	0/2	0/2
O121	+	0/2	0/2
O145	+	0/4	0/4
O157	+	28/28	0/28

* Strains positive for the *eae* gene were further tested using primer sets P1EH–P2EH and P40–P20.

† EPEC O55.H$^-$ and H7 strains but no H6, H34 and H44 strains yielded a specific amplification product.

‡ All VT-positive O111.H$^-$ and H8 but no VT-positive O111.H11 strains yielded a specific amplification product. EPEC strains of serotypes O111.H$^-$ and O111.H2 did not yield a specific amplification product.

the C1–C2 probe (data not shown). The P40–P20 probe also did not hybridize with EPEC strains which were *eae* positive as determined with the C1–C2 probe (data not shown, see below).

Allele specific PCR

The same primer sets were used in PCR assays to test strains from *eae* positive VTEC serogroups (Table 3). Primers P1EH and P2EH produced amplicons only from VT-positive O157.H$^-$, O157.H7 and VT-negative O55.H$^-$ and O55.H7 strains. All other *eae* positive strains gave negative results when tested with these allele specific primers. Similarly, primers P40 and P20 produced amplicons only from VT-positive O111.H$^-$ and O111.H8 strains. All other *eae* positive strains gave a negative result with these primers. The sizes of specific amplification products obtained in the PCR studies are shown in Table 2. Lack of a specific product of the expected molecular size was considered as negative. The specificity of amplification products was confirmed by probing them with labelled oligonucleotide primers A2 and B1 (Fig. 2). Oligonucleotide A2 derived from a site on the 3′ end of an O157.H7 *eae* gene hybridized only with the 0·45 kb amplification product of VT-positive O157.H7, O157.H$^-$ strains, and O55.H7 (Fig. 2, lanes 1, 5 and 6 respectively) and O55.H$^-$ strains. The 0·40 kb amplification product of O111.H8 (lane 8) and O111.H$^-$ (lanes 10–11) strains hybridized with the internal probe B1 derived from a site on the 3′ end of an O111.H8 *eae* gene.

Eae *distribution in VT-negative isolates of serogroups O55 and O111*

Because of our interest in the O157/O55 and the O111 alleles we also studied VT-negative isolates of serogroups O55 (24 strains) and O111 (12 strains) in more detail. Verotoxin negative isolates of the following O55 serotypes contained *eae*

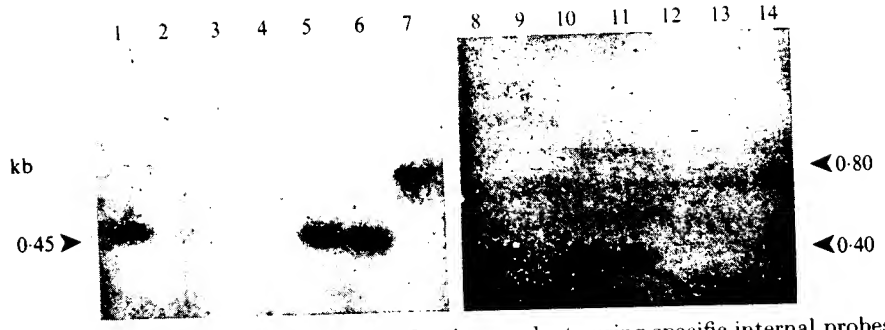

Fig. 2. Southern blot analysis of amplification products using specific internal probes A2 based on O157 sequence (lanes 1–7) and B1 based on O111 sequence (lanes 8–14). Probe A2 hybridized with a 0·45 kb product amplified from serotypes O157.H7 (lane 1), O157.H$^-$ (lane 5), O55.H7 (lane 6) using primers P1EH–P2EH and with a 0·8 kb product from serotype O157.H7 using primers S1–S2 (lane 7). A2 did not hybridize with amplification products of serotype O111.H8 using primers P40–P20 (lane 2) and serotype O40.H8 using primers P1EH–P2EH (lane 3) or a 1·1 kb product of serotype O157.H7 using primers C1–C2 (lane 4). Probe B1 hybridized with a 0·40 kb product amplified from serotypes O111.H8 (lane 8) and O111.H$^-$ (lanes 10–11) using primers P40–P20, and a 0·8 kb product from serotype O111.H8 using primers S1–S2 (lane 14). B1 did not hybridize with amplification products of serotypes O111.H11 (lane 9), O5.H$^-$ (lane 12) and O26.H11 (lane 13) using primers P40–P20.

alleles: O55.H$^-$ (4/7), O55.H6 (1/1), O55.H7 (7/7), O55.H34 (1/1) and O55.H44 (3/3). Of these only O55.H$^-$ (4/7) and O55.H7 (7/7) hybridized with the specific probe P1EH–P2EH from the 3′ end of the O157.H7 allele (Table 3). Serotypes O55.H$^-$ (3/7), O55.H1 (1/1), O55.H2 (1/1), O55.H4 (1/1), O55.H11 (1/1) and O55.H21 (1/1) did not hybridize with the conserved C1–C2 probe. For VT-negative isolates of serogroup O111, serotypes O111.H$^-$ (4/4) and O111.H2 (2/2) hybridized with the C1–C2 probe while serotypes O111.H12 (0/4) and O111.H21 (0/2) did not. None of the VT-negative O111 strains hybridized with the O111.H8 allele specific probe P40–P20 (Table 3). Representative strains (27 isolates) of the classical EPEC serotypes as defined by WHO were also tested. All EPEC strains of serotypes O26.H$^-$ (2), O26.H11 (2), O86.H$^-$ (2), O114.H2 (2), O119.H6 (2), O127.H$^-$ (2), O127.H6 (1), O128ab.H2 (1), O142.H6 (2), O158.H23 (1) and 1/2 O86.H34 strains hybridized with the C1–C2 probe, whereas strains of serotypes O86.H2 (2), O125.H21 (1), O126.H27 (2), O127.H9 (1) and O127.H21 (2) did not. None of the *eae* positive strains hybridized with the allele specific probes P1EH–P2EH or P40–P20.

DISCUSSION

Many, but not all VTEC strains possess the *eae* gene. Our results extend the findings of Willshaw and colleagues and add VTEC of serotypes O111.H8, O111.H11, O118.H16, O118.H30 and O121.H19 to the group known to contain *eae* homologues [35]. Except for non-motile strains, all isolates of a single O.H serotype were concordant with regard to the presence or absence of the *eae* gene in both studies. This is an example of the previously recognized association of specific virulence genes with individual O.H serotypes [36, 37]. Non-motile strains of serogroups O145 and O111ac which differ with respect to *eae* status, may be derivatives of different H serotypes.

All studies of VTEC colonization in animal models such as gnotobiotic piglets, calves and rabbits have shown adherence by the classical AE lesion [22–24, 38–40]. Barrett and colleagues found that non-O157 VTEC isolates from humans were more likely to be *eae* positive than were animal isolates, and suggested that the *eae* gene is an important virulence marker for human disease [41]. However, they did not study strains isolated from cattle which were being investigated for severe diarrhoea and HC. In the present study 30 cattle with severe diarrhoea or HC were infected with *eae* positive strains of serotypes O5.H$^-$, O26.H11, O103.H2, O111.H$^-$, O111.H8, O111.H11 and O118.H16 whereas only six symptomatic cattle were infected with *eae* negative strains. Faecal cultures from asymptomatic cattle yielded 15 *eae* positive strains (nine of which were serotype O157.H7 and six were of other serotypes) and 22 *eae* negative strains of various serotypes. The isolation of O157.H7 strains from asymptomatic cattle has been noted before [18, 19, 42]. This indicates that disease in cattle is more likely to be associated with *eae* positive organisms of specific serotypes and that the *eae* gene in these strains may be a potential virulence marker.

It appears that while the *eae* gene is carried by a group of VTEC which are particularly virulent in humans and cattle, its presence *per se* is not predictive of virulence of a particular strain in a specific animal host. Whereas the majority (82%) of isolates from humans with HUS or bloody diarrhoea were *eae* positive, important exceptions included VTEC of serotypes O18.H7, O91.H21, O113.H21, O117.H4 and O145.H$^-$. With the exception of serotype O91.H21, these serotypes were not represented among the VTEC strains reported by Willshaw and colleagues [35]. It is noteworthy that *E. coli* associated with oedema disease of swine do not produce A/E lesions [43], and do not contain the *eae* gene [44]. Thus the *eae* gene is clearly not necessary for production of the VTEC-associated systemic complications of HUS in humans, and oedema disease of swine. Additional studies using animal models will be required to further characterize adherence patterns of *eae* negative VTEC in the gastrointestinal tract.

The nucleotide sequence of the O55.H7 allele was 99·8% similar to that of the O157.H7 allele at the 3′ end (Fig. 1) raising the possibility that these strains share some common evolutionary origin. Whittam and colleagues [45], using multilocus enzyme electrophoresis have demonstrated that EPEC of serotypes O55.H7 are very closely related to VTEC of serotypes O157.H7. The nucleotide sequence variation at the 3′ end of the *eae* alleles allowed us to construct probes and PCR primer sets which specifically detected VTEC of serotypes O157.H7 and O157.H$^-$ strains. These reagents also yielded positive results with EPEC of serotypes O55.H7 and O55.H$^-$ but were negative when tested against all other EPEC strains including other O55 serotypes. It is possible that they might also detect VTEC of serotype O55.H7, but such strains have not been isolated in North America to date (H. Lior, unpublished data). The O157 *eae* primers 19–20 reported by Gannon and colleagues detected O157, O55 and O145 strains [44]. However, primer 20 is based on a sequence, 153 bp downstream of the termination codon of the *eae* allele of serotype O157.H7.

A second probe and set of PCR primers specifically detected VTEC of serotypes O111.H8 and O111.H$^-$ but not VTEC of serotype O111.H11 nor *eae* positive

EPEC strains of serogroup O111. The O111ab lipopolysaccharide (LPS) of EPEC is antigenically different from the O111ac LPS characteristic of VTEC, suggesting that there are significant differences between EPEC and VTEC of serogroup O111 [35, 46].

Our studies were extended to VT-negative isolates of serogroups O55 and O111. Whereas O55.H$^-$, O55.H6 and O55.H7 are considered to be classical EPEC serotypes, some authors feel that virtually all H-types of serogroup O55 are associated with diarrhoea [37, 47]. Hybridization studies demonstrated *eae* alleles in O55 isolates of serotypes H$^-$, H6 and H7 (as expected) as well as H34 and H44. It seems possible that serotypes O55.H34 and O55.H44 should be classified as EPEC on this basis. However, further studies would be required to demonstrate intestinal colonization and AE lesion formation in animal models and epidemiologic association with diarrhoea in humans. Other EPEC O55 serotypes, and serotypes O111.H12 and O111.H21 strains were *eae* negative. Strains of serotype O111.H21 and a single isolate of O55.H4 have previously been shown to exhibit enteroaggregative *E. coli* (EAggEC) adherence characteristics and to hybridize with the EAggEC probe [48, 49].

In conclusion, the study of *eae* gene distribution will be a useful method for subclassification of EPEC and VTEC. The mechanism of adherence of *eae* negative VTEC and EPEC needs to be studied. There is clearly significant variation in the 3′ end of *eae* alleles which might allow the development of serotype-specific diagnostic tools. Use of allele-specific probes and PCR primers may be of help in identifying small numbers of VTEC of specific serotypes in cultures of animal and human faeces and food samples.

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(FILE 'HOME' ENTERED AT 13:17:05 ON 08 MAR 2002)

FILE 'MEDLINE, BIOSIS, CAPLUS, EMBASE, SCISEARCH' ENTERED AT 13:17:18 ON 08 MAR 2002

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L2 0 S MARTIN?/AU AND BACON?/AU AND TYLER?/AU AND MUNRO?/AU
L3 192 S MARTIN?/AU AND BACON?/AU
L4 0 S L3 AND COLI
L5 41 S SHIGA? AND COLI AND MARTIN?/AU
L6 19 S 1995/PY AND L3
L7 19 DUP REM L6 (0 DUPLICATES REMOVED)
L8 7 S 1995/PY AND L5
L9 82 S 1995/PY AND MARTIN I/AU
L10 35 DUP REM L9 (47 DUPLICATES REMOVED)
L11 1 S 1995/PY AND MARTIN I?/AU AND COLI

FILE 'STNGUIDE' ENTERED AT 13:26:47 ON 08 MAR 2002

FILE 'MEDLINE, BIOSIS, CAPLUS, EMBASE, SCISEARCH' ENTERED AT 13:37:06 ON 08 MAR 2002

L12 0 S MICROBIOL?/SO AND (MARTIN/AU OR BACON/AU OR TYPLER/AU OR MUNR
L13 15762 S MICROBIOL?/SO AND (MARTI?/AU OR BACON?/AU OR TYLER?/AU OR MUN
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L15 138 S L14 AND 1995/PY
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L17 6 DUP REM L16 (10 DUPLICATES REMOVED)

FILE 'STNGUIDE' ENTERED AT 13:40:30 ON 08 MAR 2002

FILE 'MEDLINE, BIOSIS, CAPLUS, EMBASE' ENTERED AT 13:53:22 ON 08 MAR 2002

L18 51 S ALIGN? AND (VEROTOXIN OR 0-157 OR O-157 OR O157 OR 0157)
L19 36 DUP REM L18 (15 DUPLICATES REMOVED)
L20 64 S (SHIGA? OR VEROTOXIN?) AND (CONSERVE? OR ALIGN?) AND (0157 OR
L21 25 DUP REM L20 (39 DUPLICATES REMOVED)
L22 57 S L20 NOT L19
L23 18 S L21 NOT L19

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